Methyl paraben-Toxicity & Teratogenicity studies in Avian Embryos-
FDA Contract #72-345 No Date

J20

METHYL PARABEN

TOXICITY and TERATOGENICITY STUDIES in Avian Embryos

FDA Contract #72-345

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GENERAL PROCEDURES

The protocols as specified under FDA Contract #72-345 were followed in the investigation of toxicity and potential teratogenicity of the specified substance. The toxicity of the substance was evaluated from the percentage hatch of embryos injected either in the air cell or yolk at either zero hours (pre-incubation) or after 96 hours incubation to provide four separate evaluations.

EGG SOURCE AND HANDLING

All eggs used in these investigations were from Shaver Starcross pullets housed at the Poultry Research Center of the University of Arizona in Tucson. The parent stock was maintained on the University of Arizona breeder diet which had been formulated to provide more than adequate amounts of all the known nutrients required by the breeding hen.

The feed was specially prepared to assure no contaminations and did not contain any additive drugs such as antibiotics. All eggs prior to use (within 48 hours of lay) were candled to remove any containing blood spots, abnormal air cells or abnormal shells, and only clean eggs ranging in weight from 23 - 26 ounces per dozen were used.

The supply flock was tested to assure the absence of Pullorum and Mycoplasma gallisepticum.

The eggs were incubated in forced draft Jamesway 252 machines with automatic temperature and humidity controls and an automatic turning device.

COMPOUND HANDLING FOR INJECTION

The substance tested was solubilized in a number of the prescribed solvents in order to determine the maximum concentrations which could be employed. Where possible, water was the solvent of choice. Maximum

STUDIES on the TOXICITY and TERATOGENICITY of METHYL PARABEN in AVIAN EMBRYOS

SUMMARY and CONCLUSIONS

Absolute alcohol solutions of methyl paraben were employed in the four test protocols for the evaluation of toxicity and teratogenicity. Methyl paraben was embryo toxic under the conditions of these studies when injected in the air cell at levels of 50 mg/kg and above in fertile eggs prior to incubation and at levels of 25 mg/kg employing air cell administration in eggs containing 96 hour embryos. This substance was considerably more toxic when yolk administration was employed. Levels of 5 mg/kg and higher elicited a significant increase in embryo mortality ($P < 0.01$). Significant linear regressions between log dose and probit of mortality incidences were obtained for the air cell-0 hr and yolk-96 hr series.

The administration of methyl paraben up to 250 mg/kg did not significantly increase the incidence of abnormal embryos or H-L-S-V abnormalities when the individual dose level responses were evaluted by chi-square analyses. Chi-square tests for all dose levels in comparison with the solvent controls yielded significant values ($P < 0.01$) for the 0-hr injection times for both yolk and air cell administration routes. These data suggest that methyl paraben may be teratogenic to chicken embryos under the conditions employed.

injection volume was 0.05 ml. and all solvents and glassware were autoclaved prior to preparation of the solutions for use. The dose levels were administered with a microliter syringe using sterilized needles.

The preliminary range-finding studies using each of the administration routes and times were carried out with 10 - 25 eggs per dose level and included solvent controls, untreated controls and either drilled or pierced controls.

The actual dose-response protocol was carried out in two or more injections on different days to produce a minimum of 100 eggs at each dose level in five or more levels selected from the range- finding studies.

EXAMINATIONS OF EMBRYOS AND CHICKS

Eggs were candled daily and the dead embryos removed, examined and any abnormalities recorded. Five chicks from each dose level in each hatch were X-rayed to determine any skeletal abnormalities. Additional eggs injected at the approximate LD-50 level and an additional level below that were incubated and embryos at 8, 14, 17 days and hatch chicks removed for histopathological examinations.

In additional studies representative chicks from the dose-response protocol were saved. These chicks were housed in electrically-heated battery brooders with raised wire floors and fed University of Arizona diets. Feed consumption and growth rates were evaluated at 6 weeks of age and a sample of the birds sacrificed for gross and histopathological examinations.

DATA HANDLING

All data were coded on forms provided by FDA for computer input. In addition to summaries of mortalities and abnormalities, a number of statistical evaluations were carried out. These statistical analyses included the following for both mortality and the incidence of abnormal embryos:

1. Chi-square tests for all dose levels and for each level against the solvent control.
2. Linear regression analyses + chi square test of linearity.
   a. % response against dose
   b. % response against log dose
   c. log % response against dose
   d. arcsin transformation against dose
   e. arcsin transformation against log dose
3. Log dose against Probit using Finney's maximum likelihood method.
   a. Where significant, the LD-30, 50, 70 and 90's were estimated with 95% confidence intervals.
4. One-way analyses of variance.
5. Linear regression with replication.

Methyl paraben was solubilized in absolute alcohol for use in the four test protocols. The maximum dose level employed was 250 mg/kg (12.5 mg/egg).

MORTALITY

The mortality data obtained in the four test protocols are shown in Tables 1 - 4. The results of these tests suggest that methyl paraben was embryo toxic at the higher dose levels employed. Chi-square analyses indicated significantly higher embryo mortalities ($P<0.01$) when methyl paraben was administered in the air cell at 0 hrs at doses of 50 mg/kg and above (Table 5).

Air cell administration of methyl paraben in fertile eggs containing 96 hr embryos produced increased embryo death rates at 25 mg/kg and higher.

In the yolk 0-hr administration series none of the individual dose levels ranging from 4 - 250 mg/kg elicited a significant increase in embryo mortality when tested by chi-square; however, the combination of all dose levels in this series produced a significant chi-square value ($P< 0.005$). Yolk injections employing 96 hr embryos indicated a higher degree of toxicity for methyl paraben since significant increases in embryo mortality were obtained at levels of 5 mg/kg and above.

Probit analyses of the mortality data yielded statistically significant linear regressions between log dose and probit of mortality for only the air cell-0 hr and yolk-96 hr series. LD-50 estimates from these analyses were 67 mg/kg for the air cell-0 hr injected embryos and 5.6 mg/kg for the 96-hr embryos receiving methyl paraben in the yolk (Table 6). These data strongly suggest that methyl paraben was embryo toxic under the conditions of these studies.

TERATOLOGY

The incidences of abnormal embryos and those showing head, skeletal and visceral abnormalities are enumerated in Tables 1 - 4. Chi-square analyses of these data failed to indicate a significant increase in abnormality incidence for any of the dose levels tested with the exception of the 50 and 100 mg/kg levels administered in the yolk prior to incubation (Table 7). These two levels were employed in the range-finding studies and contained only 20 eggs at each dose level (Table 3). When the number of abnormalities occurring at all dose levels was evaluated by chi-square, the 0-hr series for both air cell and yolk administration of methyl paraben was significant at the 0.005 level of probability. These data strongly suggest that, although there was no linear relationship between log dose and probit, the injection of methyl paraben into chicken embryos did increase the incidence of abnormalities. A similar evaluation results from the analyses of H-S-L-V abnormalities (Table 9).

The individual teratological findings resulting from methyl paraben injection are shown in Table 10.

TABLE 2

METHYL PARABEN
In Alcohol, Dehydrated

Air Cell - 96 Hrs

Dose, ppm	No. Fertile	Mortality %	Mortality #	Abnormal Total %	Abnormal Total #	Abnormal H-S-V-L %	Abnormal H-S-V-L #	Abnormalities by category: Head %	Head #	Skeletal %	Skeletal #	Viscera %	Viscera #	Limbs %	Limbs #	Structural %	Structural #	Toxic Response %	Toxic Response #	Functi %
250	20	100.00	20	0.00	0	0.00	0													
200.0	20	90.00	18	0.00	0	0.00	0													
100.0	20	100.00	20	0.00	0	0.00	0													
50.0	20	100.00	20	0.00	0	0.00	0													
25.0	119	28.57	34	2.52	3	1.68	2	0.84	1			0.84	1			0.84	1			
20.0	117	48.71	57	0.85	1	0.00	0									0.85	1			
10.0	140	37.85	53	1.42	2	0.71	1							0.71	1			0.71	1	
5.0	110	37.27	41	5.45	6	8.18	9	1.81	2			1.81	2	4.54	5					
2.0	38	92.10	35	0.00	0	0.00	0													
1.0	116	23.27	27	1.72	2	0.86	1	0.86	1									0.86	1	
0.0	168	42.26	71	1.78	3	1.78	3	1.19	2			0.59	1					0.59	1	
drilled	140	12.14	17	0.71	1	1.42	2	0.71	1			0.71	1			0.71	1			
untreated	398	13.56	54	0.75	3	0.25	1	0.25	1							0.25	1			0.50

SUMMARY - ALL DOSE LEVELS

720	45.14	325	1.94	14	1.81	13	0.56	4		0.42	3	0.83	6	0.28	2	0.28	2	

TABLE 3

METHYL PARABEN
In Alcohol, Dehydrated

Yolk - 0 Hrs

Dose, ppm	No. Fertile	Mortality %	Mortality #	Abnormal Total %	Abnormal Total #	Abnormal H-S-V-L %	Abnormal H-S-V-L #	Abnormalities by category: Head %	Head #	Skeletal %	Skeletal #	Viscera %	Viscera #	Limbs %	Limbs #	Structural %	Structural #	Toxic Response %	Toxic Response #	Functic %
250.0	20	40.00	8	5.00	1	5.00	1							5.00	1					
200.0	122	50.81	62	1.63	2	1.63	2	0.81	1			0.81	1			0.81	1			
120.0	104	39.42	41	0.96	1	0.96	1			0.96	1									
100.0	20	25.00	5	10.00	2	5.00	1							5.00	1	5.00	1			
60.0	104	34.61	36	0.00	0	0.00	0													
50.0	20	40.00	8	10.00	2	15.00	3	5.00	1			5.00	1	5.00	1					
20.0	104	29.80	31	0.96	1	0.96	1					0.96	1							
10.0	17	11.76	2	0.00	0	0.00	0													
5.00	144	29.86	43	0.69	1	0.00	0											0.69	1	
4.00	34	55.88	19	0.00	0	0.00	0													
0.0	116	38.79	45	0.00	0	0.00	0													
pierced	121	27.27	33	0.82	1	0.82	1					0.82	1							
ntreated	398	13.56	54	0.75	3	0.25	1	0.25	1							0.25	1			0.50

SUMMARY - ALL DOSE LEVELS

689	37.01	255	1.45	10	1.31	9	0.29	2	0.15	1	0.44	3	0.44	3	0.29	2	0.15	1	

TABLE 1

METHYL PARABEN
in Alcohol, Dehydrated

Air Cell - 0 Hrs

Dose, ppm	No. Fertile	Mortality %	Mortality #	Abnormal Total %	Abnormal Total #	Abnormal H-S-V-L %	Abnormal H-S-V-L #	Abnormalities by category: Head %	Head #	Skeletal %	Skeletal #	Viscera %	Viscera #	Limbs %	Limbs #	Structural %	Structural #	Toxic Response %	Toxic Response #	Functic %
250.0	20	100.00	20	0.00	0	0.00	0													
200.0	125	100.00	125	0.00	0	0.00	0													
120.0	104	96.15	100	0.96	1	1.92	2	0.96	1					0.96	1					
100.0	20	85.00	17	10.00	2	15.00	3	10.00	2			5.00	1							
60.0	104	46.15	48	0.00	0	0.00	0													
50.0	20	65.00	13	10.00	2	10.00	2					5.00	1	5.00	1					
20.0	104	25.96	27	0.00	0	0.00	0													
10.0	18	27.77	5	0.00	0	0.00	0													
5.0	60	18.33	11	0.00	0	0.00	0													
4.0	38	36.84	14	5.26	2	5.26	2	2.63	1			2.63	1							
0.0	157	20.38	32	1.91	3	1.91	3	0.63	1	0.63	1	0.63	1							0.63
drilled	122	13.93	17	2.45	3	0.81	1					0.81	1					0.81	1	1.63
ntreated	398	13.56	54	0.75	3	0.25	1	0.25	1							0.25	1			0.50

SUMMARY - ALL DOSE LEVELS

613	61.99	380	1.14	7	1.47	9	0.65	4			0.49	3	0.33	2				

TABLE 4

METHYL PARABEN
in Alcohol, Dehydrated

Yolk - 96 Hrs

se, om	No. Fertile	Mortality %	#	Abnormal Total %	#	H-S-V-L %	#	Abnormalities by category: Head %	#	Skeletal %	#	Viscera %	#	Limbs %	#	Struc-tural %	#	Toxic Response %	#	Functional %	#
0	20	90.00	18	10.00	2	10.00	2	5.00	1			5.00	1					5.00	1		
0	20	100.00	20	0.00	0	0.00	0														
0	20	80.00	16	0.00	0	0.00	0														
0	19	89.47	17	0.00	0	0.00	0														
0	97	87.62	85	2.06	2	1.03	1	1.03	1							1.03	1				
0	100	97.00	97	0.00	0	0.00	0														
0	118	74.57	88	2.54	3	1.69	2					1.69	2							0.84	1
0	98	61.22	60	2.04	2	1.02	1					1.02	1			1.02	1	1.02	1		
0	37	32.43	12	0.00	0	0.00	0														
0	100	28.00	28	5.00	5	4.00	4	2.00	2			1.00	1	1.00	1	2.00	2	4.00	4		
0	156	32.05	50	4.48	7	3.20	5	1.28	2			0.64	1	1.28	2	0.64	1	1.28	2	0.64	1
d	119	21.00	25	1.68	2	1.68	2					0.84	1	0.84	1	0.84	1			0.84	1
ted	398	13.56	54	0.75	3	0.25	1	0.25	1							0.25	1			0.50	2

SUMMARY - ALL DOSE LEVELS

	629	70.11	441	2.23	14	1.59	10	0.64	4			0.79	5	0.16	1	0.64	4	0.95	6	0.16	1

TABLE 5

METHYL PARABEN

Chi-Square Analyses of Mortality

Dose Level mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
1.000	-	10.121* (less)	-	0.300
2.000	-	28.853*	-	0.023
4.000	3.731	-	2.479*	-
5.000	0.022	0.496	1.908	19.693*
10.000	0.179	0.446	3.631	46.913*
20.000	0.817	0.916	1.581	102.499*
25,000	-	5.053*	-	72.017*
50.000	16.348*	21,601*	0.021	21.269*
60.000	18.354*	-	0.251	-
100.000	33.843*	21.601*	0.866	15.404*
120.00	140.674*	-	0.002	-
200.00	175.548*	14.479*	3.007	31.331*
250.00	50.432*	21.601*	0.021	22.724*
All Doses (DF)	353.161*(10)	164.787*(10)	27.257*(10)	238.179*(10)

*Probability < 0.05 - 0.005

TABLE 6

METHYL PARABEN

Probit Analyses - Mortality

	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
LD-30	55.5	NS	NS	2.1
LD-50	67.0	NS	NS	5.6
LD-70	80.9	NS	NS	14.8
LD-90	106.1	NS	NS	60.3

TABLE 7

METHYL PARABEN

Chi-Square Analyses of Abnormalities

Dose Level mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
1.000	-	0.177	-	0.013
2.000	-	0.006	-	0.678
4.000	0.361	-	0.000	-
5.000	0.183	1.805	0.012	0.460
10.000	0.135	0.042	0.000	0.275
20.000	0.680	0.021	0.003	3.080
25.000	-	0.000	-	0.440
50.000	1.795	0.117	5.883*	0.104
60.000	0.680	-	0.000	-
100.000	1.795	0.117	5.883*	0.129
120.000	0.009	-	0.003	-
200.000	0.940	0.117	0.455	0.129
250.000	0.088	0.117	1.000	0.265
All Doses (DF)	34.508* (10)	10.791 (10)	31.361* (10)	13.852 (10)

* Probability < 0.05 - 0.005.

TABLE 8

METHYL PARABEN

Probit Analyses - Abnormalities

	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
	NS	NS	NS	NS

TABLE 9

METHYL PARABEN

Chi-Square Analyses of HLSV Abnormalities

Dose Level mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
1.000	-	0.105	-	0.034
2.000	-	0.058	-	0.279
4.000	0.845	-	0.000	-
5.000	0.007	2.933	0.000	0.478
10.000	0.475	0.025	0.000	0.158
20.000	0.185	0.214	0.003	1.809
25.000	-	0.026	-	0.463
50.000	2.803	0.439	5.883*	0.004
60.000	0.185	-	0.000	-
100.000	2.803	0.439	1.000	0.010
120.000	0.131	-	0.003	-
200.000	0.305	0.439	0.455	0.010
250.000	0.379	0.439	1.000	0.733
All Doses (DF)	37.671*(10)	17.875(10)	27.697*(10)	14.698(10)

*Probability $< 0.05 - 0.005$.

TABLE 10
Methyl Paraben in Alcohol, Dehydrated

TERATOGENIC FINDINGS

TREATMENT	TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL	SPECIFIC FINDINGS NO.	DESCRIPTION
Control	398	3	1	Exencephaly
			1	Ataxia
			1	Cachexia; clubbed down
Drilled - 0 hrs	122	3	1	Celosomia - abdomen
			2	Cachexia; hypopigmentation (1)
Drilled - 96 hrs	140	1	1	Anophthalmia; exencephaly; abnormal shortening
				maxilla; dysgnathia - beak; dwarfism; celosomia
				abdomen
Pierced - 0 hrs	121	1	1	fusion failure - abdomen
Pierced - 96 hrs	119	2	1	congenital malformation - left foot
			1	cachexia; aplasia-down; fusion failure - abdomen
Air Cell - 0 hrs 120.0 mg/kg	104	1	1	buphthalmia; abnormal shortening - maxilla; cong malformation - left foot
100.0	20	2	2	dysgnathia - beak; celosomia - abdomen (1)
50.0	20	2	1	congenital abnormal curvature - toe
			1	congenital fusion failure - abdomen
4.0	38	2	1	celosomia - abdomen
			1	exencephaly
0.0	157	3	1	cachexia
			1	fusion failure - abdomen
			1	exencephaly; thoracopagus

TERATOGENIC FINDINGS

TREATMENT	TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL	SPECIFIC FINDINGS	
			NO.	DESCRIPTION
Air Cell - 96 hrs 25.0 mg/kg	119	3	1	celosomia - abdomen
			1	dwarfism
			1	anophthalmia; exencephaly; abnormal shortening -maxi
20.0	117	1	1	agenesis - down
10.0	140	2	1	displacement - hindlimb
			1	edema
5.0	110	6	1	phocomelia - hindlimb, celosomia - abdomen
			2	malrotation - hindlimb
			1	agenesis - toe
			1	dysplasia - eyelid; displacement - toe
			1	anophthalmia; dysgnathia - beak; celosomia - abdomen
1.0	116	2	1	edema
			1	acrania
0.0	168	3	1	edema
			1	dysplasia - eyelid
			1	dysplasia - eyelid; celosomia - abdomen

TERATOGENIC FINDINGS

TREATMENT		TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL	SPECIFIC FINDINGS NO.	DESCRIPTION
Yolk - 0 hrs	250.0 mg/kg	20	1	1	malformation - foot
	200.0	122	2	1	dysgnathia - beak; dwarfism
				1	celosomia - abdomen
	120.0	104	1	1	malformation - pelvis
	100.0	20	2	1	cachexia
				1	malformation - foot
	50.0	20	2	1	exencephaly; celosomia - abdomen
				1	malformation - foot
	20.0	104	1	1	celosomia - abdomen
	5.0	144	1	1	hemorrhage
olk - 96 hrs	250.0	20	2	1	abnormal flexion - maxilla; edema
				1	fusion failure - abdomen
	25.0	97	2	1	dysgnathia - beak
				1	dwarfism
	10.0	118	3	2	fusion failure - abdomen
				1	cachexia
	5.0	98	2	1	dwarfism; hypopigmentation - down
				1	celosomia - abdomen

TERATOGENIC FINDINGS

REATMENT		TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL	SPECIFIC FINDINGS NO.	DESCRIPTION
- 96 hrs	1.0 mg/kg	100	5	2	dwarfism; hypopigmentation - down
				1	anophthalmia; abnormal shortening maxilla; celosomia - abdomen
				1	hemorrhage
				1	abnormal shortening - maxilla; agenesis - wing; edema
	0.0	156	7	1	anophthalmia; dysgnathia - beak
				1	cachexia; agenesis - toe
				1	celosomia - abdomen
				1	abnormal shortening - maxilla; agenesis - mandible
				1	dwarfism
				2	edema
				1	malrotation - hindlimb